INFECTION AND IMMUNITY, Jan. 1996, p. 355–357
0019-9567/96/$04.00+0

Vol. 64, No. 1

Protective Immunity to Shiga-Like Toxin I following Oral Immunization with Shiga-Like Toxin I B-Subunit-Producing *Vibrio cholerae* CVD 103-HgR

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Received 9 June 1995/Returned for modification 31 July 1995/Accepted 12 October 1995

This study addresses a mechanism for inducing systemic immunity to Shiga-like toxins by oral administration of a Shiga-like toxin I B-subunit-expressing *Vibrio cholerae* vaccine strain [CVD 103-HgR(pDA60)]. Two sets of three rabbits were given either CVD 103-HgR or CVD 103-HgR(pDA60) orally. All rabbits immunized with CVD 103-HgR(pDA60) developed neutralizing serum antibodies to Shiga-like toxin I. None of the controls developed such antibodies.

Shiga-like toxins (SLTs) (also known as verotoxins), which were named after the prototype toxin produced by *Shigella dysenteriae* type 1, are produced by *Escherichia coli* and are well-documented causes of morbidity throughout the world. SLT-producing bacteria are now a major cause of acute renal failure in children in the United States. These bacteria have been associated with bloody and nonbloody diarrhea and with systemic complications such as hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (8, 12, 16). Over 80 serotypes of SLT-producing *E. coli* have been identified in humans, and the production of SLTs is the one feature that they all share. Free SLTs are detectable in the stools of patients infected with SLT-producing *E. coli* (2, 13). These facts, combined with the proven in vitro cytotoxic effects of SLTs on intestinal epithelial and various endothelial cells, which are considered major targets of the toxins in vivo (11, 18, 21), suggest that SLTs are critical virulence factors in both gastrointestinal and systemic disease.

SLTs can be broadly divided into two groups: SLT-I, whose amino acid sequence differs by only three nucleotides from that of the Shiga toxin produced by *S. dysenteriae* type 1 strains, and SLT-II, which is about 60% homologous with SLT-I at the amino acid level (10). All SLTs are heterodimeric nonglycosylated proteins consisting of an enzymatic A subunit, which inactivates the 60S ribosomal subunit and inhibits protein synthesis, and a pentamer of binding B subunits (5, 9, 19, 22). We previously cloned and hyperexpressed the SLT-I B subunit in both *E. coli* and *Vibrio cholerae* (1), and expressed the SLT-II B subunit in *E. coli* (3).

Currently the only therapy available for patients infected with SLT-producing *E. coli* or suffering from the systemic effects of the toxins, such as hemolytic uremic syndrome, is supportive. While control measures, in relation to food preparation and cooking, are the most appropriate means of preventing SLT-induced disease, there is a need for alternative strategies such as the development of active therapy and vaccines which could prevent SLT-related hemolytic uremic syndrome. It is clear from animal studies that the presence of serum antibodies to the toxins offers protection against toxin-induced disease (7, 23). Purified SLT-I B subunit has been shown to induce a protective immune response when given parenterally to rabbits (17) and may be a suitable antigen for use as a human vaccine to prevent the local and systemic complications caused by SLT-producing *E. coli.*

In view of current trends towards enteral vaccine delivery and multicomponent vaccines, the objective of the present study was to use the *V. cholerae* vaccine strain (CVD 103-HgR) to present SLT-I B subunit to the gut mucosa by the oral route and then to determine whether an immune response to the foreign antigen was elicited. CVD 103-HgR is a recombinant derivative of *V. cholerae* O1 strain 569B in which 94% of *ctxA1* has been deleted and a 4.2-kb fragment encoding resistance to mercury has been inserted into the *hylA* locus (15). The SLT-I B-subunit-expressing plasmid (pDA60) was constructed by subcloning a portion of the *sltI* gene containing DNA encoding the entire SLT-I B-subunit gene and the carboxy-terminal 18 amino acids encoded by the A subunit gene into pKK223-3 (Pharmacia LKB Biotechnology, Piscataway, N.J.) under the control of a *tac* promoter. pDA60 was inserted into CVD 103-HgR by electroporation. Six male New Zealand White rabbits (approximately 7 weeks old) were anesthetized with xylazine and ketamine after a 12-h fasting period. The rabbits were then each given 50 mg of cimetidine intravenously. After 15 min, a stomach tube was inserted in each rabbit and 10 ml of a 10% sodium bicarbonate solution was administered, followed by the bacterial inoculum (10^{10} to 10^{11} organisms); 30 min later they were each given 1 ml of tincture of opium intraperitoneally. Three rabbits received the parental strain, CVD 103-HgR, and three were given CVD 103-HgR(pDA60). All rabbits were given a second oral inoculation of the same bacteria 20 days after the first inoculation. Blood was drawn from the rabbits prior to the experiment, 12 days after the first inoculation, and 14 days after the second inoculation. Approximately 5 weeks after the second inoculation, enterotoxicity responses to both cholera toxin and various doses of SLT-I were assessed in the six rabbits, as previously described (14).

Sera were tested for the presence of immunoglobulin G antibodies to SLT-I B subunit by coating enzyme-linked immunosorbent assay (ELISA) plates (Nunc Maxisorp) with 5 μg of recombinant SLT-I B subunit per ml (1). Various dilutions of serum were added, bound antibodies were detected with a

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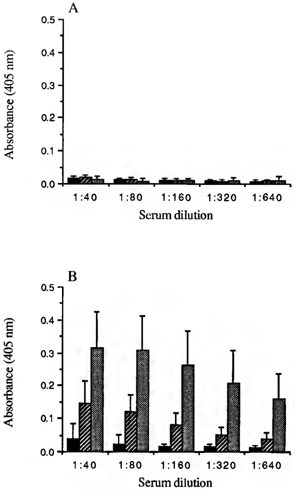

FIG. 1. ELISA for the detection of immunoglobulin G anti-SLT-I B-subunit antibodies in various dilutions of rabbit serum from control animals given CVD 103-HgR (A) and animals given CVD 103-HgR(pDA60) (B). Blood was tested prior to immunizations (solid bars), after the first immunization (hatched bars), and after the second immunization (cross-hatched bars). Data are means + standard errors of the means for three rabbits in each group.

goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate followed by Sigma 104 phosphatase substrate, and the results were expressed as A_{405}. Having already established that a concentration of 1 ng of SLT-I per ml killed approximately one-half of the HeLa cells in a well of a 96-well plate (data not shown), we determined the neutralizing capacity of the serum antibodies to SLT-I by incubating various concentrations of serum with SLT-I (1 ng/ml) for 1 h at room temperature before adding the toxin-serum mixture to HeLa cells grown in 96-well plates. Following overnight incubation at 37°C (5% CO_2), the plates were washed, the remaining attached cells were stained with crystal violet as described (6), and the results were expressed as percent HeLa cell survival compared with survival of non-toxin-treated control cells.

We have previously found that *V. cholerae* CVD 103-HgR(pDA60) produces large amounts of SLT-I B subunit in vitro, and we have been able to purify over 10 mg of SLT-I B subunit per liter of culture (4). The results from the present experiments demonstrate that there was little difference among preimmune sera from the animals and that none of the animals immunized with CVD 103-HgR developed an antibody response to SLT-I B subunit. In contrast, there was evidence of antibodies to SLT-I B subunit in the animals immunized with CVD 103-HgR(pDA60) after the first immunization. The level of antibody, as determined by the ELISA, rose further after the second immunization (Fig. 1). Similar results were observed in the neutralization experiments (Fig. 2). In contrast, there was a clear serum vibriocidal antibody response in all six rabbits after the second immunization. The titers in the three rabbits given CVD 103-HgR were 160, 320, and 20,480, and the titers in the three given CVD 103-HgR(pDA60) were 160, 320, and 640. None of the rabbits had antibodies to the B subunit of cholera toxin in the preimmune serum; however, five of the six rabbits, including all those given CVD 103-HgR(pDA60), developed a significant antibody response to the B subunit of cholera toxin after the second immunization. To maximize the data from each animal, we also determined the effects of immunization on toxin-induced intestinal fluid secretion (20). Ileal loops were constructed and inoculated with buffer as a negative control, with cholera toxin as a positive control, or with various doses of SLT-I. Of the six rabbits, two (both of

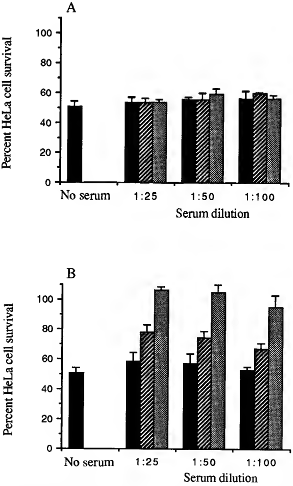

FIG. 2. Capacity of various dilutions of rabbit serum to neutralize the effects of SLT-I on HeLa cells. Results for control animals given CVD 103-HgR (A) and animals given CVD 103-HgR(pDA60) (B) are shown. Blood was tested prior to immunizations (solid bars), after the first immunization (hatched bars), and after the second immunization (cross-hatched bars). Data are means + standard errors of the means for the three rabbits in each group. HeLa cell survival is calculated as a percentage of the survival of cells grown in the absence of toxin (high survival values denote low levels of toxin).

which received CVD 103-HgR) died during the experiment. The four remaining animals [one given CVD 103-HgR and three given CVD 103-HgR(pDA60)] were sacrificed 18 h after inoculation. There was little difference in response to cholera toxin among the four animals (3.5, 2.8, 3.2, and 3.0 ml of fluid per cm of ileum in the rabbit given the parental strain and the three immunized with the SLT-I B-subunit-producing strain, respectively). The loops injected with 1 μg of SLT-I produced 1.4, 0.3, 0.0, and 0.6 ml of fluid per cm of ileum in the rabbit given the parental strain and the three immunized with the SLT-I B-subunit-producing strain, respectively. Administration of 100 ng of SLT-I resulted in 0.8, 0.2, 0.0, and 0.0 ml of fluid per cm of ileum in the rabbit given the parental strain and the three immunized with the SLT-I B-subunit-producing strain, respectively. While the number of animals used in the study is too small for one to draw any definitive conclusions, the loop experiments suggest that the three animals given CVD 103-HgR(pDA60) were somewhat protected from the enterotoxic effects of SLT-I compared with the single control. The fact that all three animals with antibodies to SLT-I B subunit survived the SLT-I loop challenge may also be indicative of a protective response; however, this is speculative.

These data demonstrate that oral immunization of rabbits with an SLT-I B-subunit-expressing *V. cholerae* vaccine vector induces a significant neutralizing serum antibody response to SLT-I. The data also suggest the induction of a neutralizing mucosal immune response to the enterotoxic effect of SLT-I in the ileal loop model. Both effects occurred without compromising the production of vibriocidal antibodies or anti-cholera toxin B-subunit antibodies. Our findings suggest the possibility of inducing protective immunity to both the local and systemic complications associated with SLT-producing *E. coli* by oral immunization with an SLT-I B-subunit-expressing cholera vaccine strain. These results encourage the further development and testing of SLT B-subunit-based vaccines.

This work was supported by Public Health Service grants AI-16242 from the NIAID and P 30 DK-34928 for the Center for Gastroenterology Research on Absorptive and Secretory Processes from the NIDDK, Bethesda, Md., and a Health Sciences for the Tropics grant from the Rockefeller Foundation.

We thank Genevieve Losonsky for performing the anti-cholera toxin B-subunit ELISA and Sabine DeBreucker for technical assistance.

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Editor: A. O'Brien